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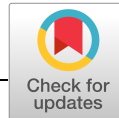
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## ORIGINAL RESEARCH ARTICLE

# Combination therapy of mesenchymal stromal cells and sulfasalazine attenuates trinitrobenzene sulfonic acid induced colitis in the rat: The S1P pathway

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## Abstract

Adipose derived mesenchymal stem cells (ASCs) transplantation is a novel immunomodulatory therapeutic tool to ameliorate the symptom of inflammatory bowel disease (IBD). The objective of this study was to investigate the therapeutic effects of combined sulfasalazine and ASCs therapy in a rat model of IBD. After induction of colitis in rats, ASCs were cultured and intraperitoneally injected ( $3 \times 10^6$  cells/kg) into the rats on Days 1 and 5 after inducing colitis, in conjunction with daily oral administration of low dose of sulfasalazine (30 mg/kg). The regenerative effects of combination of ASCs and sulfasalazine on ulcerative colitis were assessed by measuring body weight, colonic weight/length ratio, disease activity index, macroscopic scores, histopathological examinations, cytokine, and inflammation markers profiles. In addition, western blot analysis was used to assess the levels of nuclear factor-kappa B (NF- $\kappa$ B) and apoptosis related proteins in colitis tissues. Simultaneous treatment with ASCs and sulfasalazine was associated with significant amelioration of disease activity index, macroscopic and microscopic colitis scores, as well as inhibition of the proinflammatory cytokines in trinitrobenzene sulfonic acid (TNBS)-induced colitis. Moreover, combined ASCs and sulfasalazine therapy effectively inhibited the NF- $\kappa$ B signaling pathway, reduced the expression of Bax and prevented the loss of Bcl-2 proteins in colon tissue of the rats with TNBS-induced colitis. Furthermore, combined treatment with ASCs and sulfasalazine shifted inflammatory M1 to anti-inflammatory M2 macrophages by decreasing the levels of MCP1, CXCL9 and increasing IL-10, Arg-1 levels. In conclusion, combination of ASCs with conventional IBD therapy is potentially a much more powerful strategy to slow the progression of colitis via reducing inflammatory and apoptotic markers than either therapy alone.

## KEYWORDS

adipose derived mesenchymal stem cells, inflammation, low-dose sulfasalazine, NF- $\kappa$ B

## 1 | INTRODUCTION

Inflammatory bowel diseases (IBDs) are chronic, relapsing, inflammatory disorders of the gastrointestinal tract which include ulcerative colitis (UC) and Crohn's disease. These two manifestations of IBD are a global health concern considering their growing incidence that affects millions of people worldwide (M'koma, 2013). To date, the etiology and pathogenesis of chronic IBD are still poorly understood. However, it seems that dysregulation of mucosal immune reaction against intestinal microflora and excessive mucosal damage caused by specific intestinal antigens contributes to the pathogenesis of chronic IBD (Wallace, Zheng, Kanazawa, & Shih, 2014). Various compounds and approaches, such as 5-aminosalicylates (5-ASA), sulfasalazine, corticosteroids, immunosuppressive agents or selective biological inhibitors of inflammatory pathways, including tumor necrosis factor  $\alpha$  (TNF- $\alpha$ ), have been traditionally used for medical treatments of IBD. However, the limited effectiveness of long-term treatment, undesirable side effects, inconvenient dosing regimen of the conventional drugs and the frequent failure to induce and maintain remission have limited their extensive application (Staveland et al., 2015).

Stem cell-based therapy, as a promising alternative strategy, has recently attracted considerable attention for the treatment of IBD (Duran & Hommes, 2016). Mesenchymal stem cells (MSCs), which are multipotent cells that can be easily isolated from various tissues and are capable of differentiating into cells of mesodermal lineage, such as bone, cartilage, muscle, and fat, are considered as an attractive candidate for cell therapy. The capacity of MSCs to differentiate into diverse cell lineages demonstrates that these cells have the potential to promote healing of the damaged tissues or organs (Robinson et al., 2014; Shirian et al., 2016). In addition, MSCs exert immunomodulatory and anti-inflammatory effects by decreasing the production of inflammatory cytokine and the number of inflammatory cells, such as DCs, natural killer cells (NKs), T and B cells (Kyurkchiev et al., 2014; Zhang et al., 2009). Therefore, hundreds of clinical trials are being carried out using MSCs for treatment of several immune mediated conditions, including Graft vs Host Disease (GVHD), aplastic anemia, Crohn's disease, rheumatoid arthritis, and multiple sclerosis (Zhao, Ren, & Han, 2016).

Combining cell therapy and low doses of traditional medications, such as transplantation of MSC combined with administration of sulfasalazine, can provide improved therapeutic outcome with fewer side effects compared with long-term administration of high doses of conventional medications.

In the present study, MSCs, derived from adipose tissue, have been used due to the ease of accessibility and their higher suppression effects on inflammatory cells than other sources of MSCs, such as bone marrow, amnion, and umbilical cord (Ivanova-Todorova et al., 2009).

Sulfasalazine is selected as the reference drug in most of the studies conducted on colitis. Moderate to high doses of sulfasalazine (100–500 mg/kg) are usually applied as the positive control. The aims

of the present study were to investigate the therapeutic effects of combination of low dose of sulfasalazine (30 mg/kg) and adipose derived mesenchymal stem cells (ASCs) in a trinitrobenzene sulfonic acid (TNBS)-induced IBD rat model as well as the capacity of this combination therapy to restore immune tolerance and inhibit the inflammatory responses *in vivo*.

Our hypothesis was that ASCs transplantation together with administration of low dose of sulfasalazine would result in an increase in regenerative responses of each of the treatments alone. The results of the present study indicated that the combination therapy synergistically enhances colon regeneration, reduces active inflammation, and improves overall colon function compared with either of the treatments alone.

## 2 | MATERIALS AND METHODS

### 2.1 | Isolation and expansion of adipose-MSCs

The human ASCs were isolated from lipoaspirates obtained from human adipose tissue of healthy adult donors. Tissues were washed with PBS before being digested with collagenase type I (0.1%) in PBS. An equal volume of Dulbecco's modified Eagle's medium (DMEM; Gibco-Invitrogen, Carlsbad, CA) containing 10% fetal bovine serum (FBS; Gibco-Invitrogen) was added to the collagenase type I solution to stop collagenase activity and the mixture was centrifuged at 1,000g for 5 min to obtain a cell pellet which was then resuspended in 1 ml of expansion medium (DMEM/F12 supplemented with 100 U/ml penicillin–streptomycin, 1% glutaMAX (Gibco-Invitrogen), and 20% FBS (MSC-qualified; Gibco). The isolated cells were seeded into the culture flasks containing expansion medium at a density of 100,000 cells/cm<sup>2</sup>. The culture medium was replaced every 24 hr for a period of 3 days to get rid of nonadherent contaminating cells.

### 2.2 | Flow cytometric analysis

The cell surface marker profile of ASCs was investigated by flow cytometry analysis. Approximately,  $5 \times 10^5$  cells at passage 2 to 6 were incubated with specific PE- or FITC-conjugated mouse antibodies against human CD11b, CD34, CD44, CD90, CD105 (eBioScience, San Diego, CA), and subjected to flow cytometric analysis using a Beckman Colter flow cytometer and FACScan program (eBioScience).

### 2.3 | Animals

The experimental protocols were approved by the Animal Care and Use Committees of the Tehran University of Medical Sciences (TUMS). All animal procedures were approved by the ethical committee of the TUMS. Ten-week-old male Wistar rats (180–220 g) were obtained from the animal house of the School of Medicine, TUMS (Tehran, Iran). Three rats were housed per cage in a temperature-controlled room (20–24°C) with a 12-hr light/dark

cycle. All the rats had access to standard laboratory chow and water ad libitum.

## 2.4 | TNBS-induced colitis model

To induce colitis, TNBS (Sigma-Aldrich, Deisenhofen, Germany) was dissolved in ethanol 50% to reach a concentration of 40 mg/kg and was intrarectally administered 8 cm proximal to the anus (total volume of 300  $\mu$ l) using a lubricated silicone catheter (Rashidian et al., 2016). For TNBS administration, rats were anesthetized with diethyl ether. The animals were maintained in a head side down position for 1 min to prevent any solution leakage.

## 2.5 | Experiment design

The animals were randomly assigned to seven groups, each consisting of six rats and the following treatments were performed for each group:

1. Control group (50% ethanol + normal saline).
2. TNBS group (TNBS + normal saline).
3. Sulfasalazine group (TNBS + sulfasalazine 30 mg/kg/day).
4. ASCs group (TNBS + ASCs).
5. ASCs + sulfasalazine 30 group (TNBS + ASCs + sulfasalazine 30 mg/kg/day).

The animals were orally administered with normal saline and sulfasalazine. Moreover, ASCs were intraperitoneally injected on Days 1 and 5 postcolitis induction (cells were suspended in phosphate buffered saline [PBS],  $1 \times 10^6/0.3$  ml/rat).

## 2.6 | The disease activity index (DAI)

The severity of the induced colitis was assessed using the DAI. To this end, the animals were weighed and the stool consistency as well as the presence and degree of occult or gross rectal bleeding were recorded on a daily bases. The DAI was determined, as previously described (El-Salhy & Umezawa, 2016), by rating the percentage of the body weight loss (0, *no body weight loss*; 1, 1–5%; 2, 6–10%; 3, 11–15%; and 4, >15%), stool consistency (0, *normal*; 2, *loose*; 4, *diarrhea*) and the degree of rectal bleeding (0, *normal*; 2, *occult bleeding*; and 4, *gross bleeding*). The DAI was assessed by averaging these three separate scores.

## 2.7 | Colon macroscopic damage and area of colonic lesions

Severity of the induced colitis was evaluated by an independent observer blind to the identity of the treatments according to a scale ranging from 0 to 4 as follows: 0, *no macroscopic changes*; a, *mucosal erythema only*; b, *mild mucosal edema, slight bleeding or small erosions*; c, *moderate edema, bleeding ulcers or erosions*; and d, *severe ulceration, erosions, edema, and tissue necrosis* (Millar et al., 1996).

## 2.8 | Histological evaluation and immunohistochemistry (IHC)

The colon samples were embedded in paraffin and cut into 5  $\mu$ m sections which were then deparaffinized, cleared, and rehydrated in a graded ethanol series. The sections were stained with hematoxylin and eosin and scored (Ren, Yuan, Zhang, Wei, & Wang, 2015) considering the following parameters: (a) inflammation (0, none; 1, slight; 2, moderate; 3, severe); (b) extent of injury (0, none; 1, mucosa; 2, mucosa and submucosa; 3, transmural); (c) crypt damage (0, none; 1, basal 1/3 damage; 2, basal 2/3 damage; 3, only surface epithelium lost; 4, entire crypt and epithelium lost), and (d) percent involvement ( $\times 1$ : 0–25%,  $\times 2$ : 26–50%,  $\times 3$ : 51–75%,  $\times 4$ : 76–100%). The total score was the sum of all the above parameters. For evaluation of myeloperoxidase (MPO) using IHC, paraffin-embedded sections (4  $\mu$ m) of the colon tissue were deparaffinized in xylene, washed in a graded ethanol series, and rehydrated in PBS. Antigen retrieval was performed in citrate buffer (pH 6.0) for 15 min at 3°C using a steamer. The nonspecific binding sites were blocked by incubating the tissue sections in a 3% bovine serum albumin solution for 1 hr at 37°C followed by incubation of the samples with the primary antibody against MPO (rabbit polyclonal to MPO; 1:100; Abcam, Cambridge, MA) at 4°C, overnight. The tissue sections were then incubated with goat-anti-rabbit IgG (HRP-conjugate; 1:100; Abcam) for 1 hr at 37°C. No washing step was performed between incubation with the primary and secondary antibodies. Finally, the slides were stained with diaminobenzidine (Boster Biological Technology Ltd., Wuhan, China) at room temperature for 10 min and the staining process was stopped with distilled water. The prepared tissue sections were then counterstained with hematoxylin for 5 min, dehydrated in alcohol and xylene, and covered with neutral balsam.

## 2.9 | Western blot analysis

The colon tissue samples were homogenized in standard radio-immunoprecipitation assay buffer. The supernatant was removed after centrifugation at 12,500g for 20 min at 4°C. Total protein concentration was determined using a NanoDrop 2000C (Thermo Fisher Scientific, Waltham, MA). Equal amounts (100  $\mu$ g) of proteins were separated from each sample using 10% sodium dodecyl sulfate polyacrylamide gel electrophoresis and transferred to poly vinylidene fluoride membranes.

The membranes were blocked with 5% nonfat dry milk and 0.1% Tween-20 in Tris-buffered saline for 1 hr at room temperature, followed by incubation with rabbit anti-NF- $\kappa$ B p65 (1:500 dilution), Bcl-2 (1:5,000 dilution), Bax (1:5,000 dilution), and anti- $\beta$ -actin (1:500 dilution) antibodies (Abcam) at 4°C overnight. After being washed with Tris-buffered saline Tween-20, the membranes were incubated with a secondary goat-anti-rabbit IgG (HRP-conjugate; 1:10,000; Abcam) antibody at room temperature for 1 hr. A western blot detection system (West-Zol® Plus, iNtRON Biotechnology, Daejeon, Korea) was used to detect the target proteins on the membranes, according to the instruction of the manufacturer, before being exposed to the X-ray films.

(Bio-Rad, Hercules, CA). Density of the protein bands was quantified using Quantity One® 1-D analysis software (Bio-Rad).

## 2.10 | Enzyme-linked immunosorbent assay

The expression levels of TNF- $\alpha$ , IL-6, and IL-1 $\beta$  proteins in the colon tissue lysates were determined using the ELISA kits as described by the manufacturer (R&D Systems, Minneapolis, MN).

## 2.11 | Gene expression analysis using real-time quantitative

Total RNA was extracted using the RNeasy Mini Kit (QIAGEN AB, Sollentuna, Sweden). cDNA was synthesized using the SuperScript TMII enzyme (18064-014; Invitrogen) and quantitative real-time polymerase chain reaction (qRT-PCR) was performed using Fast SYBR Green Master Mix (Applied Biosystems, Foster City, CA) applying the primers detailed in Table 1. The thermal cycling conditions consisted of an initial denaturation step at 95°C for 5 min, 35 cycles of 94°C for 15 s, 55°C for 15 s, and 72°C for 15 s, followed by a final extension step at 72°C for 10 min. GAPDH was chosen as a housekeeping gene and used as an internal comparator in parallel with the control sample.

## 2.12 | Statistical analysis

All the values are expressed as mean  $\pm$  standard deviation. The data were analyzed by GraphPad Prism, version 5.04 (GraphPad Software Inc., San Diego, CA). Differences between the groups were evaluated using an analysis of variance, followed by the Tukey's post-hoc test.  $p < 0.05$  was considered statistically significant.

**TABLE 1** Primers applied for real-time PCR

Genes	Primer sequence	Amplicon size (bp)
IL-10	Forward: 5'-GGGAAGCAACTGAACTTCG-3' Reverse: 5'-GCTTTCGAGACTGGAAGTGG-3'	330
MCP-1	Forward: 5'-TGCTGTCTCAGCCAGATGCAGTTA-3' Reverse: 5'-AGAAGTGCTTGAGGTGGTTGTGGA-3'	356
CXCL9	Forward: 5'-GACTCCAGCACGGTGACTTA-3' Reverse: 5'-ATGCAGGAGCATCGCTGATT-3'	136
ARG1	Forward: 5'-TTGGAACGAAACGGAAGGT-3' Reverse: 5'-TGTTCCGTTTGCTGTGATGC-3'	113
FoxP3	Forward: 5'-AGAGTGCTAGAGCCAAGTGC-3' Reverse: 5'-GGCCACTGTTAGGGTATGGG-3'	286
Sphingosine kinase	Forward: 5'-CTCTTCCGAGCAGAAAGGGAA-3' Reverse: 5'-AGTGTGCAGCTGCTTATCGG-3'	290
Sphingosine-1-phosphate lyase	Forward: 5'-CTGCACCAAATACGAGCCCT-3' Reverse: 5'-TGGCTTTGGTAAGCTGCTGT-3'	188
GAPDH	Forward: 5'-GTCGGTGTGAACGGATTTG-3' Reverse: 5'-TCCCATTCTCAGCCTTGAC-3'	181

Note. PCR: polymerase chain reaction.

## 3 | RESULTS

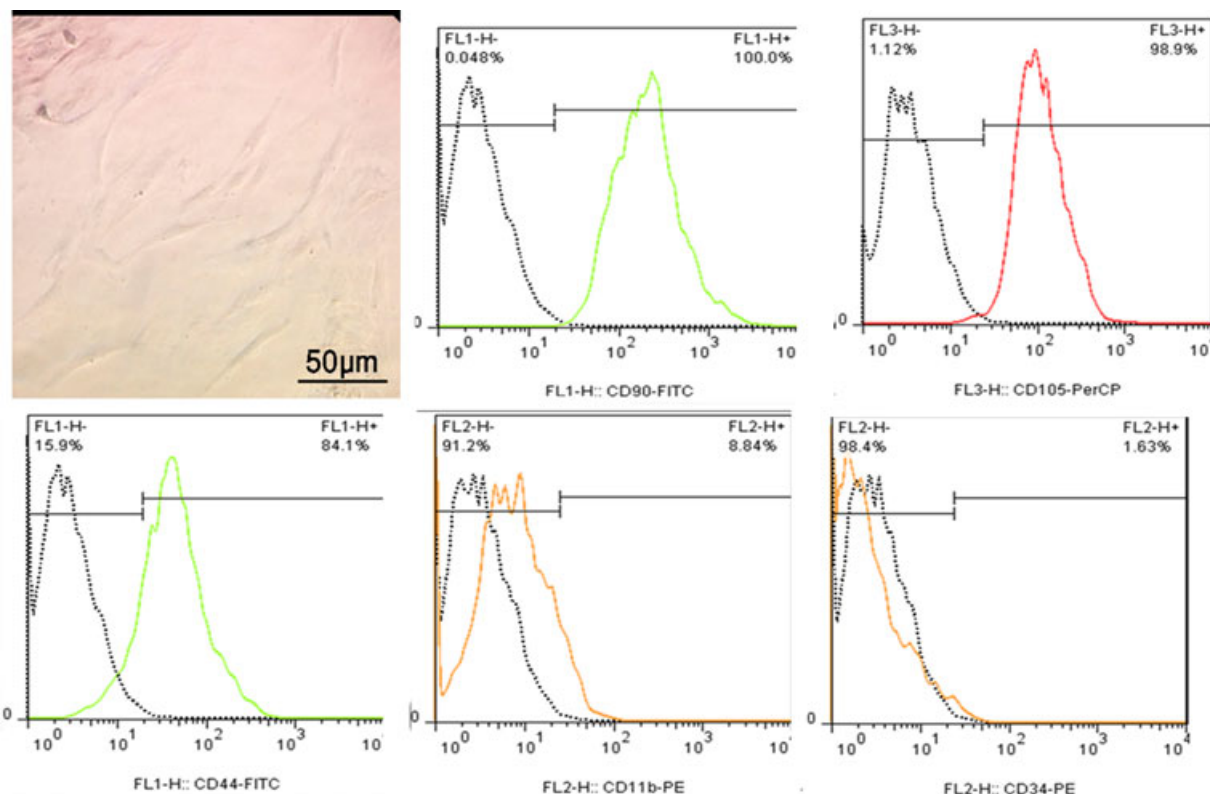
### 3.1 | Cell characteristics and phenotypic and functional validation of ASCs

MSC-specific markers, namely CD44, CD90, and D105, were strongly expressed in the ASCs whereas the expression levels of CD34 (hematopoietic progenitors) and CD11b (leukocyte common antigen) were not detectable (Figure 1a).

The fibroblastic-like ASCs grew in monolayer culture, adhered to the plastic surface and proliferated. To determine the ASCs differentiation capacity, cells were cultured in specialized culture media to induce osteogenic and adipogenic differentiation. Success of osteogenic and adipogenic differentiation was confirmed using Alizarin Red S and Oil Red O, respectively (Figure 1b).

### 3.2 | Combination treatment improves macroscopic tissue damage in TNBS-induced colitis in rats

To investigate the potential of coadministration of ASCs and low dose of sulfasalazine to attenuate colon inflammation, its efficacy was analyzed in the TNBS-induced colitis in rats as an experimental model of IBD. After intrarectal instillation of TNBS, the rats suffered a significant weight loss (Table 2) accompanied by severe bloody diarrhea, macroscopic damage score (as damage degree) and elevated colon weight to length ratio (an indicator of the ongoing colon inflammation) compared with the control rats. However, the macroscopic score and weight/length ratio of the colon were effectively improved in the ASCs-treated groups compared with that in the sulfasalazine and TNBS groups (Table 2). Nevertheless, a significant reduction in these



**FIGURE 1** Morphology, characterization and differentiation capacity of human adipose mesenchymal stem cells (ASCs). (a) Morphology of the cultured whale mesenchymal stem cells (magnifications:  $\times 100$ ) and flow cytometry analyses of ASCs cell surface markers. Positive markers (CD44, CD90, and CD105) were expressed whereas negative markers (CD34 and CD11b) were not [Color figure can be viewed at [wileyonlinelibrary.com](http://wileyonlinelibrary.com)]

parameters was observed in ASCs + sulfasalazine group compared to all the other groups (Table 2). Interestingly, we observed a similar pattern of DAI improvement and recovery in the ASCs + sulfasalazine treated group compared with all the other groups (Figure 2B).

### 3.3 | Histological improvement in response to combination treatment

Histopathological assessment of the TNBS-instilled rats included focal necrosis of the mucosa, erosion, loss of goblet cells, and submucosal edema which were characterized by high levels of inflammatory cell infiltration. Histopathological scores of the TNBS-

induced colitis were dramatically decreased in the rats treated with the combination of ASCs + sulfasalazine compared to the experimental groups treated with either sulfasalazine or ASCs. Collectively, these results suggest that ASCs and sulfasalazine provide a strong protection against TNBS-induced colonic damages (Figure 3).

TNBS instillation promoted a massive neutrophil infiltration, as revealed by the higher intracolonic MPO (a biomarker for activated neutrophils) levels observed in the colitis-induced rats compared with the healthy control group. Similarly to the colitis scores, the expression level of MPO in the ASCs + sulfasalazine-treated group was significantly lower than that detected in the experimental groups treated with either ASCs, sulfasalazine or TNBS (Figure 4).

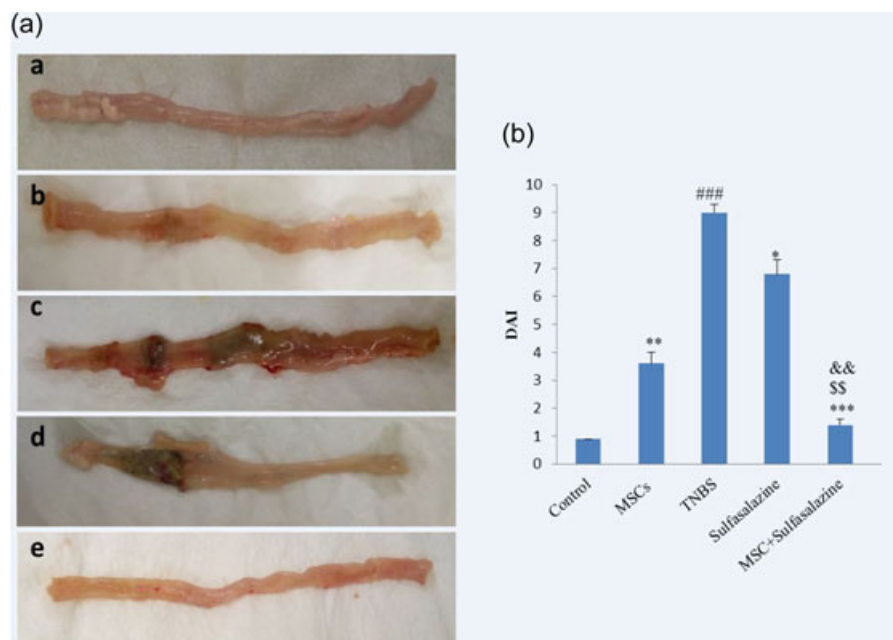
**TABLE 2** Effect of combination treatment on macroscopic parameters of colitis induced by TNBS in rats

Groups	% Change of body weight (g)	Colonic weight to length ratio (mg/cm)	Macroscopic damage (score 0–4)
Control	7.5 $\pm$ 1.6	93.7 $\pm$ 6	0
MSCs	0.8 $\pm$ 0.5**	170.63 $\pm$ 7.5**	1.4 $\pm$ 0.8*
TNBS	-20.2 $\pm$ 2.9###	296.4 $\pm$ 12.4###	3.92 $\pm$ 0.43###
Sulfasalazine	-10.6 $\pm$ 2.7	208.4 $\pm$ 11.5*	2.9 $\pm$ 0.65
MSC + sulfasalazine	3.32 $\pm$ 1.3***&\$\$	121.2 $\pm$ 4.6***&\$	0.7 $\pm$ 0.25***&\$

Note. MSCs: mesenchymal stem cells; TNBS: trinitrobenzene sulfonic acid.

\*\* $p$  < 0.01, \* $p$  < 0.05, ### $p$  < 0.001 versus control group, \*\*\* $p$  < 0.001 versus TNBS group, \$\$\$ $p$  < 0.001, versus sulfasalazine group, && $p$  < 0.01, &&& $p$  < 0.001, versus MSCs group, \$ $p$  < 0.01, \$ $p$  < 0.05, & $p$  < 0.001.





**FIGURE 2** (A) Images of the colon tissue 9 days after colitis induction: (a) control; (b) ASCs; (c) TNBS; (d) Sulfasalazine 30; (e) ASCs + sulfasalazine 30. (B) Effects of combination treatment on DAI in the experimental colitis rat model.  $^{\#}p < 0.05$ ,  $^{\#\#}p < 0.01$ ,  $^{\#\#\#}p < 0.001$  versus control group,  $^{*}p < 0.05$ ,  $^{**}p < 0.01$ ,  $^{***}p < 0.001$  versus TNBS group,  $^{\$}p < 0.05$ ,  $^{$$$}p < 0.001$  versus sulfasalazine group, and  $^{\&}p < 0.05$ ,  $^{\&\&}p < 0.01$ ,  $^{\&\&\&}p < 0.001$  versus MSCs group. ASCs: adipose derived mesenchymal stem cells; DAI: disease activity index; MSC: mesenchymal stem cells; TNBS: trinitrobenzene sulfonic acid [Color figure can be viewed at [wileyonlinelibrary.com](http://wileyonlinelibrary.com)]

### 3.4 | ASCs and inflammatory cytokine levels

To determine the immune response modulation induced by the combination ASCs and low dose of sulfasalazine, the expression levels of cytokines were analyzed in the colon tissues of colitis-induced rats. The expression levels of the cytokines, namely TNF- $\alpha$ , IL-1, IL-6, IL-17, IL-10, and TGF- $\beta$ , were measured using enzyme-linked immunosorbent assays. Although treatment with ASCs alone resulted in a significant decrease in the levels of these proinflammatory cytokines, the combination of ASCs and sulfasalazine reduced the expression levels of these cytokines to a greater extent (Figure 5). Conversely, the expression levels of IL-10 and TGF- $\beta$  (as anti-inflammatory cytokines) were increased in the colon with administration of ASCs as well as combination therapy (Figure 5).

### 3.5 | Combination treatment shifted macrophage functional phenotype in the colitis-induced colon

To investigate the effects of combination therapy on macrophage M1/M2 polarization in colonic tissues, the gene expression levels of M1 and M2 markers were assessed using qRT-PCR. The gene expression level of M1 marker, MCP-1, was significantly downregulated in all the treatment groups. However, this downregulation was grater in the ASCs + sulfasalazine group compared to that in the experimental groups treated with either sulfasalazine or ASCs (Figure 6). Conversely, the expression levels of M2 markers, such as arginase-1 (Arg-1), TGF- $\beta$ 1, and IL-10, were significantly

upregulated in ASCs-treated group compared with that in the groups treated with either TNBS or sulfasalazine. Moreover, treatment with ASCs + sulfasalazine further increased the expression levels of M2 markers (Figure 6c,d).

### 3.6 | Combination treatment increased Treg-specific gene expression

Further analyses were performed on the expression levels of the transcription factor, FoxP3, as a marker of regulatory T (Treg) cells using qRT-PCR. The expression level of FoxP3 was upregulated in the ASCs groups, treated either alone or in combination with sulfasalazine. However, treatment with ASCs + sulfasalazine significantly upregulated the expression level of FoxP3 compared to any other treatment. These results indicated that ASCs treatment increased T reg cell differentiation via upregulation of Foxp3 gene expression (Figure 7).

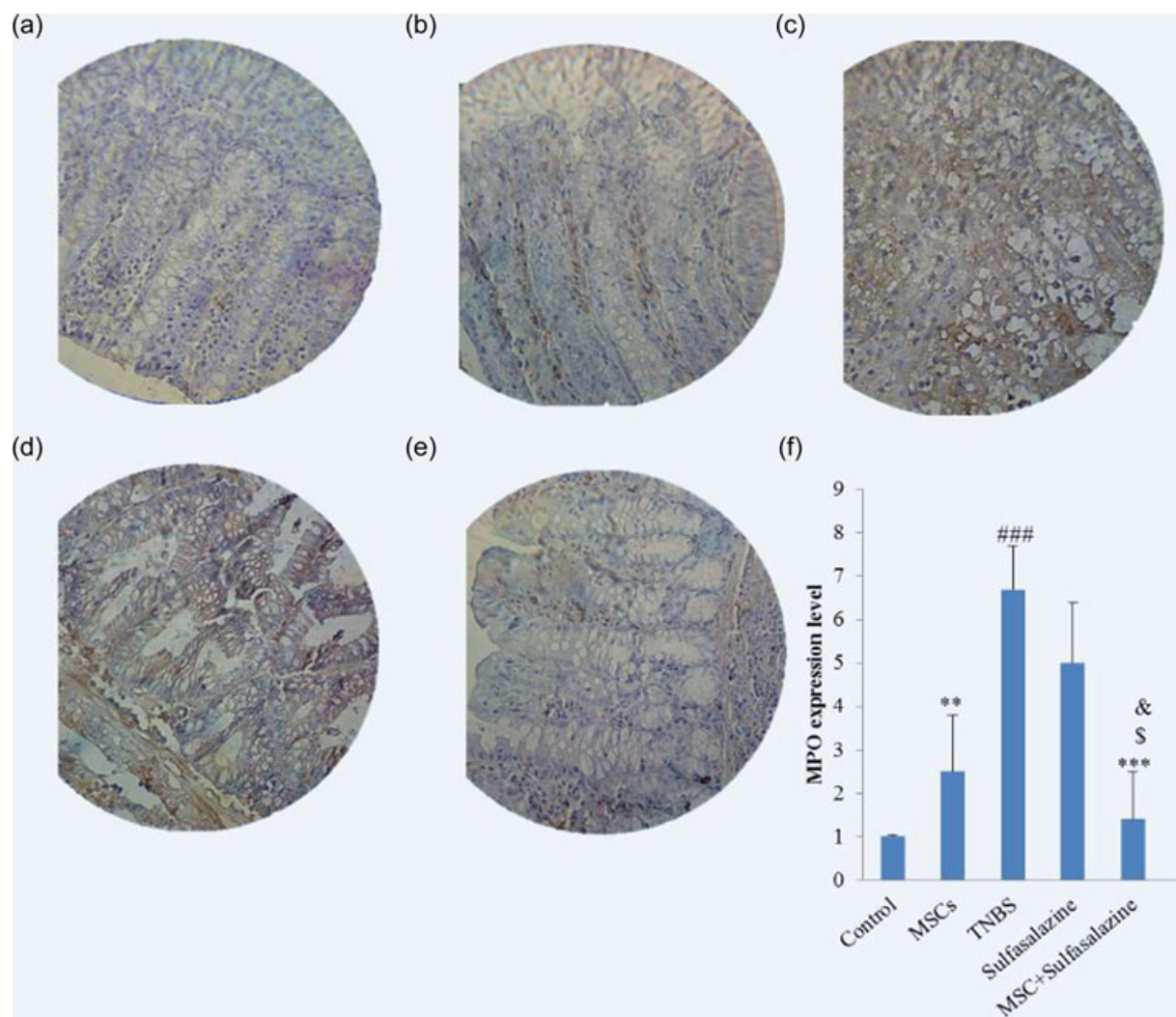
### 3.7 | Combination treatment altered the protein expression of nuclear factor-kappa B (NF- $\kappa$ B), p65, Bax and Bcl-2 in colonic tissue

Results of the western blot assay indicated that the protein expression of NF- $\kappa$ B p65 in TNBS group was markedly increased compared with the normal group ( $p < 0.01$ ). Nevertheless, both ASCs and ASCs plus sulfasalazine treatment significantly diminished the induced upregulation of NF- $\kappa$ B p65 protein expression (Figure 8).

The expression level of proapoptotic protein, Bax, was significantly increased, whereas that of the antiapoptotic protein, Bcl-2 was







**FIGURE 4** Immunohistochemical staining against MPO in colonic sections of different treatment groups ( $\times 400$ ). (a) Control. (b) ASCs. (c) TNBS. (d) Sulfasalazine 30. (e) ASC + sulfasalazine 30. (f) The expression levels of MPO-positive cells in the colon tissue. Data are expressed as mean  $\pm$  SD ( $n = 6$ ). # $p < 0.05$ , ## $p < 0.01$ , ### $p < 0.001$  versus control group, \* $p < 0.05$ , \*\* $p < 0.01$ , \*\*\* $p < 0.001$  versus TNBS group, \$ $p < 0.05$ , \$\$ $p < 0.01$ , \$\$\$ $p < 0.001$  versus sulfasalazine group. & $p < 0.05$ , && $p < 0.01$ , &&& $p < 0.001$  versus ASCs group. ASCs: adipose derived mesenchymal stem cells; MPO: myeloperoxidase; MSC: mesenchymal stem cells; TNBS: trinitrobenzene sulfonic acid; SD: standard deviation [Color figure can be viewed at [wileyonlinelibrary.com](http://wileyonlinelibrary.com)]

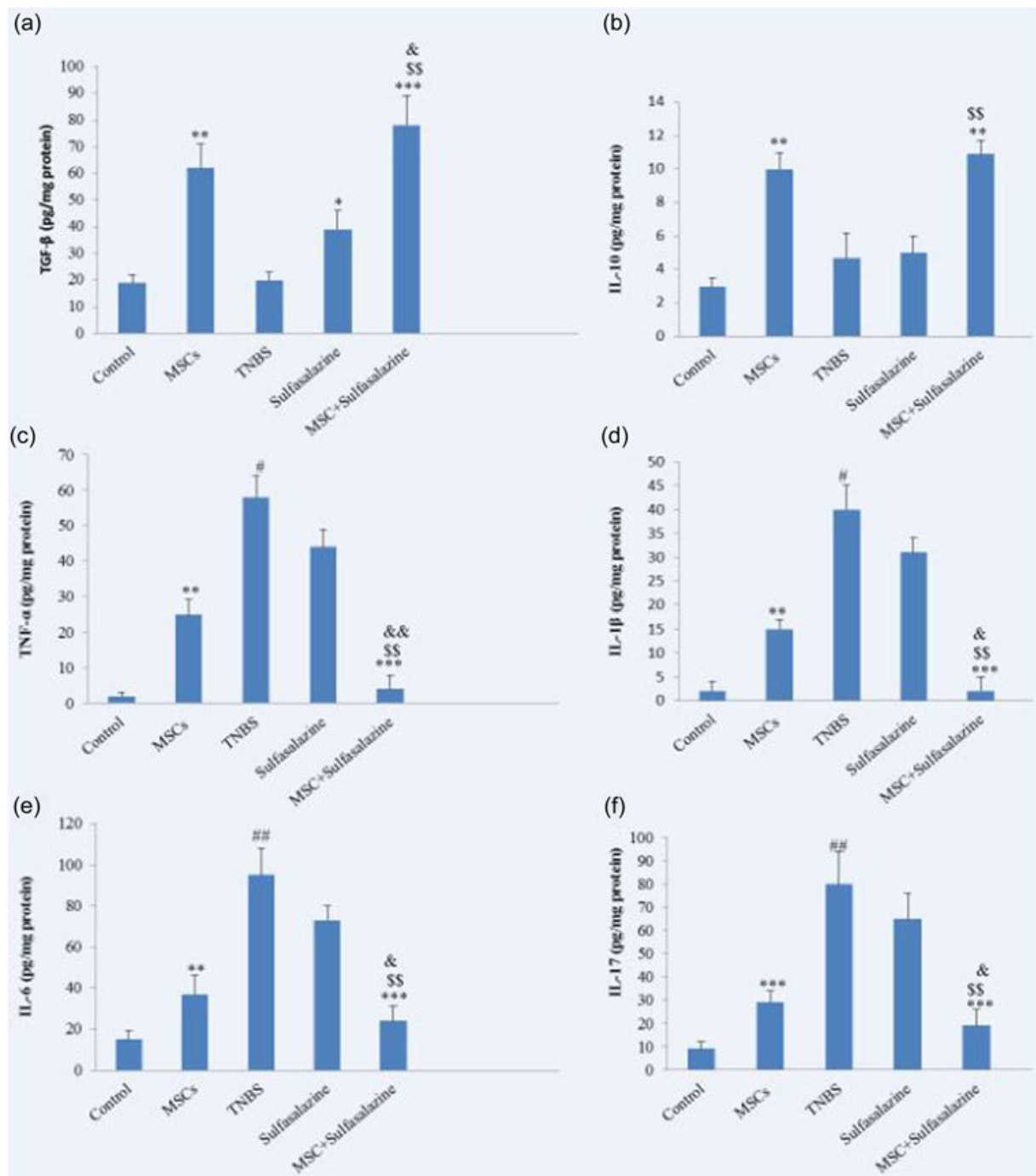
effects, including nausea, headache, and anorexia, as well as hemolysis, many of which are dose-dependent and can be avoided by lowering the dosage of sulfasalazine (Peppercorn, 1984).

Therefore, to reduce toxicity and adverse effects of conventional therapeutic approaches, it seems necessary to apply combination therapy by administering lower dosages of each drug for treatment of the disease (Sultan, Berkowitz, & Khan, 2017). In addition, combination therapy has other advantages, including increased effectiveness of treatment and greater clinical responses, as well as cost effectiveness and minimized metabolic side effects (Hanauer, 2017; Sultan et al., 2017).

The objective of this study was to evaluate the effectiveness of combination therapy with intraperitoneal injection of ASCs and sulfasalazine in a rat model of TNBS-induced colitis. TNBS-induced colitis in rat is characterized by transmural intestinal inflammation

and dysregulation of innate and adaptive immune responses that present characteristics similar to those of human IBD (Liu, Wang, & Hu, 2017).

MSCs are a heterogeneous subset of stromal cells with self-renewal ability and multipotent differentiation potential that can be isolated from fat, bone marrow, umbilical cord, amniotic fluid, placenta, and other tissues. MSCs have been demonstrated to exert potent anti-inflammatory and immunomodulating effects on almost all the cells of the innate and adaptive immune systems via direct cell-cell contact and secretion of soluble paracrine factors, such as cytokines and chemokines (Kyurkchiev et al., 2014). Previous studies have indicated that MSCs induce their therapeutic and regenerative effects on animal models of colitis by regulating and reducing the infiltration of activated immune cells at the colon site (Zhang et al., 2009). In this context, the



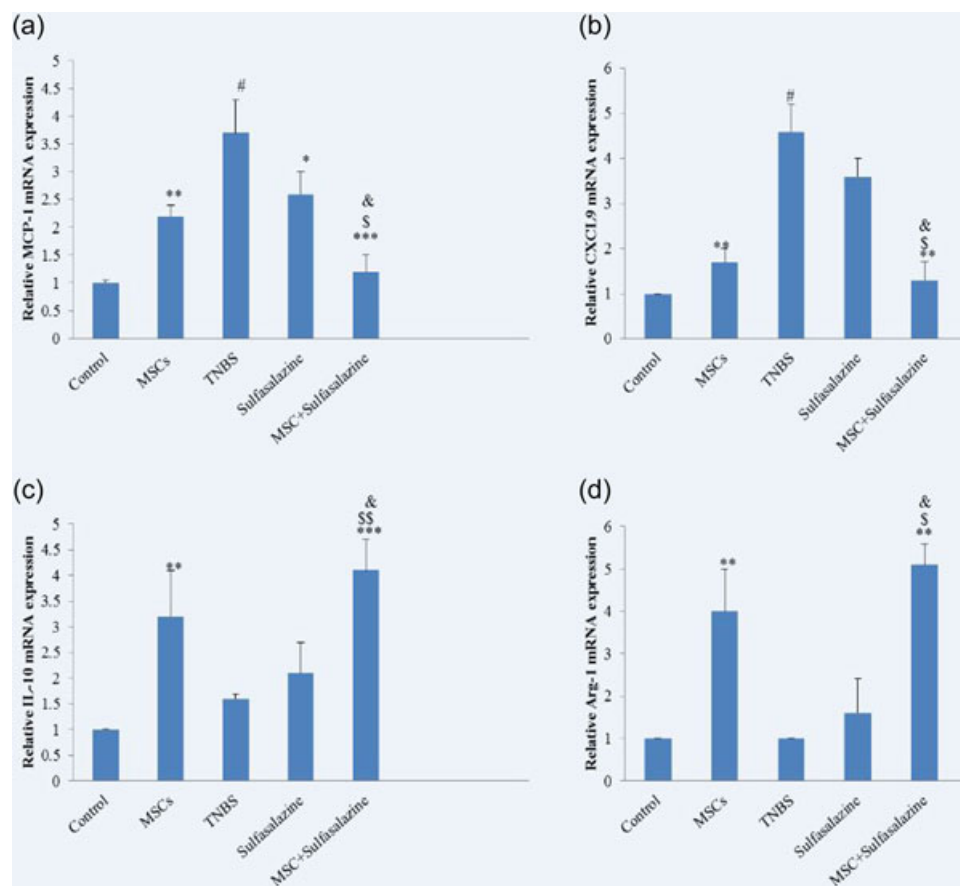
**FIGURE 5** The effects of combination treatment on the cytokines profile. The protein levels of anti-inflammatory TGF- $\beta$ 1 (a) and IL-10 (b) as well as proinflammatory TNF- $\alpha$  (c), IL-1 $\beta$  (d), IL-6 (e), and IL-17 (f) in colon extracts were measured by ELISA. # $p$  < 0.05, ## $p$  < 0.01, ### $p$  < 0.001 versus control group, \* $p$  < 0.05, \*\* $p$  < 0.01, \*\*\* $p$  < 0.001 versus TNBS group, \$ $p$  < 0.05, \$\$ $p$  < 0.01, \$\$\$ $p$  < 0.001 versus sulfasalazine group and & $p$  < 0.05, && $p$  < 0.01, &&& $p$  < 0.001 versus MSCs group. MSCs: adipose derived mesenchymal stem cells; ELISA: enzyme-linked immunosorbent assay; MSC: mesenchymal stem cells; TNBS: trinitrobenzene sulfonic acid [Color figure can be viewed at [wileyonlinelibrary.com](http://wileyonlinelibrary.com)]

advantages of ASCs over MSCs derived from other sources include easy isolation and high yield, as well as high therapeutic and immune-modulatory potentials (Melief, Zwaginga, Fibbe, & Roelofs, 2013).

In the present study, all the macroscopic and microscopic signs of inflammatory activities were significantly improved in

the ASCs + sulfasalazine group compared to the other treatment groups.

MPO is a heme enzyme that is most abundantly secreted in neutrophils and stored in their azurophilic granules. MPO is used as an indirect measure of intensity of tissue inflammation; its high level reflects an increased neutrophil infiltration as well as inflammation.



**FIGURE 6** M1 to M2 macrophage polarization. Expression levels of the macrophage phenotype-associated genes were evaluated by qRT-PCR. (a) MCP-1 and (b) CXCL9 (M1 marker), and (c) IL-10 and (d) Arg-1 (M2 marker). # $p < 0.05$ , ## $p < 0.01$ , ### $p < 0.001$  versus control group, \* $p < 0.05$ , \*\* $p < 0.01$ , \*\*\* $p < 0.001$  versus TNBS group, \$ $p < 0.05$ , \$\$ $p < 0.01$ , \$\$\$ $p < 0.001$  versus sulfasalazine group and & $p < 0.05$ , && $p < 0.01$ , &&& $p < 0.001$  versus MSCs group. ASC: adipose derived mesenchymal stem cells; MSC: mesenchymal stem cells; qRT-PCR: quantitative real-time polymerase chain reaction; TNBS: trinitrobenzene sulfonic acid [Color figure can be viewed at [wileyonlinelibrary.com](http://wileyonlinelibrary.com)]

The experimental groups treated with ASCs, especially ASCs + sulfasalazine group, presented a significantly reduced levels of MPO expression in the colonic tissue.

Production of proinflammatory cytokines, such as TNF- $\alpha$ , IL-1, and IL-6 were significantly inhibited and the protein expression levels of anti-inflammatory cytokines, including IL-10 and TGF- $\beta$  were upregulated in the colon samples of ASCs-sulfasalazine groups.

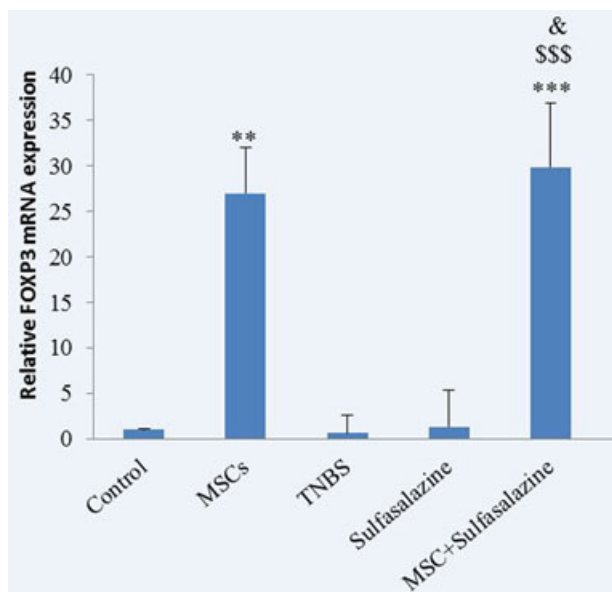
Moreover, the expression level of Foxp3, a master regulator of Treg cell development and function, was upregulated in the colon tissues of ASCs-treated groups compared with the TNBS group, which suggests that ASCs can be effectively applied in IBD treatment and they induce their effects through increasing the amounts of Treg cells. A similar result has been found when bone marrow mesenchymal stem cells were transplanted in a murine model of colitis (Chen et al., 2013).

Coadministration of ASCs and sulfasalazine downregulated the expression levels of inflammatory M1 macrophage markers, CXCL9 and MCP-1, whereas upregulated that of the anti-inflammatory M2 macrophage markers, Arg-1 and IL-10. These results are consistent with the findings of the studies conducted on animal model of IBD

that have reported a M1 to M2 macrophage phenotype switching after treatment with extracts and exosomes from MSCs (Mao et al., 2017; Song et al., 2017).

It has been demonstrated that ASCs exert protective effects against intestinal inflammation in a murine model of colitis by decreasing the number of inflammatory T cells as well as inflammatory cytokines and increasing the number of regulatory T cells as well as anti-inflammatory cytokines (Gonzalez-Rey, Gonzalez, Rico, Buscher, & Delgado, 2009). Akiyama et al. (2012) have reported that bone marrow-derived MSCs ameliorated dextran sulfate sodium (DSS)-driven colitis via activation of Fas death pathway in inflammatory T cells.

Several studies have proven that MSCs have the ability to inhibit cytotoxicity of NK cells and T cell proliferation via production of prostaglandin E2 (Németh et al., 2009), repression of differentiation of circulating T follicular helper cells by production of indoleamine 2,3-dioxygenase (François, Romieu-Mourez, Li, & Galipeau, 2012), stimulation of immunomodulatory cytokines synthesis, such as TGF $\beta$ 1, IL-10, HGF, IL-1 receptors antagonist and TNF-stimulated gene 6 proteins (TSG-6), and induction of macrophage polarization toward anti-inflammatory M2 phenotype (Zheng, Ge, Qiu, Shu, & Xu, 2015).



**FIGURE 7** The expression levels of Treg marker Foxp3 in colon tissues. <sup>#</sup> $p < 0.05$ , <sup>##</sup> $p < 0.01$ , <sup>###</sup> $p < 0.001$  versus control group, <sup>\*</sup> $p < 0.05$ , <sup>\*\*</sup> $p < 0.01$ , <sup>\*\*\*</sup> $p < 0.001$  versus TNBS group, <sup>\$</sup> $p < 0.05$ , <sup>\$\$</sup> $p < 0.01$ , <sup>\$\$\$</sup> $p < 0.001$  versus sulfasalazine group and <sup>&</sup> $p < 0.05$ , <sup>&&</sup> $p < 0.01$ , <sup>&&&</sup> $p < 0.001$  versus MSCs group. ASCs: adipose derived mesenchymal stem cells; mRNA: messenger RNA; MSC: mesenchymal stem cells; TNBS: trinitrobenzene sulfonic acid [Color figure can be viewed at [wileyonlinelibrary.com](http://wileyonlinelibrary.com)]

A recent clinical trial has reported that using placenta-derived mesenchymal-like adherent cells is a safe and efficient approach to treat IBD (Melmed et al., 2015). Another study has reported that autologous adipose-derived MSCs were able to completely heal fistula in 82% (27/33) of their patients, 8 weeks following final MSCs transplantation (Lee et al., 2013).

In recent years, investigators have shown that pretreatment of MSCs with cytokines, such as interleukin-25 (IL-25), interferon- $\gamma$  (IFN- $\gamma$ ), and TNF- $\alpha$ , as well as granulocyte colony stimulating factor (G-CSF) enhance their therapeutic effects on colitis via an anti-inflammatory mechanism (Cheng et al., 2017).

The results of the present study demonstrated that the combination cell therapy with mesenchymal stem cells and low dose of sulfasalazine could considerably be a more effective colitis therapy than either therapy alone.

Duijvestein et al. (2011) have found that MSCs phenotype, survival, differentiation, immunosuppressive capacity and in vitro function are not affected by therapeutic doses of commonly used drugs for the treatment of IBD. This suggests that coadministration of MSCs and IBD drugs can be effectively used for colitis treatment.

One of the main findings of the present study was the remarkable downregulation of sphingosine kinase (SphK) mRNA expression level in the rat model of colitis after ASCs transplantation. SphK is a key enzyme in synthesis of sphingosine-1-phosphate (S1P) which tightly regulates S1P levels in tissues and plasma. Another key regulator of sphingolipid metabolism is sphingosine-1-phosphate lyase (SPL) that catabolizes the irreversible degradation of S1P into ethanolamine

phosphate and hexadecenal (Nielsen, Li, Johansson-Lindbom, & Coskun, 2017).

S1P is a bioactive sphingolipid metabolite that is synthesized from sphingosine and ATP by SphKs and exerts diverse biological and immunologic effects. S1P, by activating STAT and NF- $\kappa$ B signaling pathways, mediates inflammatory responses and leukocyte trafficking to the sites of inflammation within the gastrointestinal tract (Wollny et al., 2017). Several studies have reported high levels of S1P expression accompanied with dysregulated of SphKs pattern in inflammation sites, such as inflamed mouse and human intestine (Crespo et al., 2017; Karupuchamy et al., 2017; Suh & Saba, 2015). On the other hand, SPL is highly expressed in normal intestine but downregulated in the colon of IBD and colorectal cancer patients as well as the murine experimental colitis (Crespo et al., 2017; Nielsen et al., 2017). In IBD, activation of STAT- and NF- $\kappa$ B-mediated pathways markedly increases the risk of malignant cell growth and tumor formation (Wollny et al., 2017). Therefore, targeting SphK/S1P axis may represent an important therapeutic approach for controlling inflammation in IBD and preventing colon carcinogenesis (Wollny et al., 2017).

A recent study has found that ASCs secretome can inhibit inflammatory responses of microglia via modulation of sphingosine kinase/S1P signaling (Marfia et al., 2016). Moreover, findings of the present study indicated that ASCs treatment reverses upregulation of SphK and downregulation of SPL in the colonic tissues of rat with TNBS-induced colitis. These results are consistent with those of the previous studies indicating that attenuation of S1P signaling via SphK inhibitors significantly abrogates colitic damage and reduces the expression levels of inflammatory molecules, IL-6 and COX-2. Moreover, it was observed that colitis severity and inflammation were enhanced by deletion of SPL after dextran sulfate sodium treatment (Degagné et al., 2014; Xi et al., 2016).

To the best of our knowledge, this is the first report demonstrating that ASCs are able to ameliorate progression of colitis by modulation of S1P signaling pathway through suppressing SphK and increasing SPL expression.

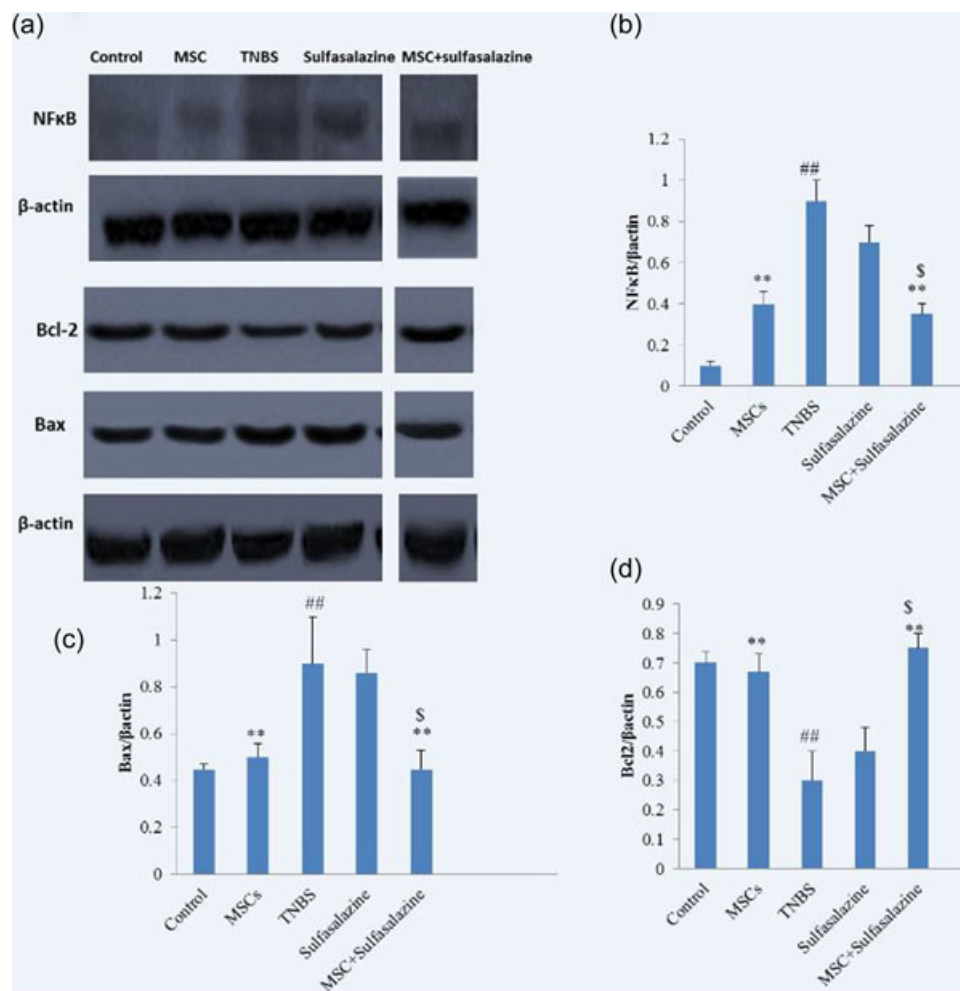
The results of this study indicated that combined administration of ASCs and low dose of sulfasalazine induces synergistic anti-inflammatory and regenerative effects on colitis.

In the present study, coadministration of ASCs and sulfasalazine significantly downregulated the expression level of NF- $\kappa$ B in the colon tissue of TNBS-induced colitis rat model.

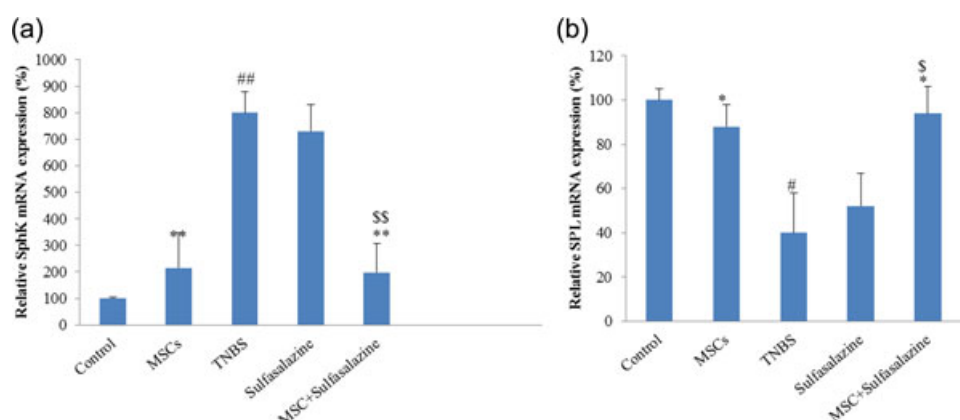
NF- $\kappa$ B, as a central transcription factor and mediator of inflammatory responses, regulates expression of many genes and plays crucial roles in immune and inflammatory reactions. Several clinical and preclinical studies have confirmed that NF- $\kappa$ B is activated in the IBD patients and the animal model of colitis and contributes to pathogenesis of IBD (Neurath, Becker, & Barbutescu, 1998; Rashidian et al., 2016; Wang, Fan, & Cao, 2016). Downregulation of the NF- $\kappa$ B activity emerges as a very attractive target for therapeutic intervention in intestinal inflammation (Neurath et al., 1998).

Increasing evidence suggests that apoptosis is one of the main mechanisms involved in colitis (Giriş et al., 2008; Gu et al., 2017; Liu,





**FIGURE 8** The protein expression levels of NF-κB, p65, Bcl-2, and Bax in colon extracts. (a) Representative western blot showing protein levels of NF-κB p65, Bcl-2, Bax, and β-actin protein. Changes in the expression level of NF-κB p65 (b), Bax (c), Bcl-2 (d) proteins after treatment. Data are expressed as mean ± SD ( $n = 6$ ). ## $p < 0.01$  versus control group, \* $p < 0.05$ , \*\* $p < 0.01$  versus TNBS group and \$ $p < 0.05$  versus sulfasalazine group. MSC: mesenchymal stem cells; TNBS: trinitrobenzene sulfonic acid; SD: standard deviation [Color figure can be viewed at [wileyonlinelibrary.com](http://wileyonlinelibrary.com)]



**FIGURE 9** Effect of combination treatment on the SphK/S1P signaling pathway-related genes. mRNA expression levels of SphK1 (a) and SPL (b) genes. # $p < 0.05$ , ## $p < 0.01$ , versus control group. \* $p < 0.05$ , \*\* $p < 0.01$ , versus TNBS group. \$ $p < 0.05$ , \$\$ $p < 0.01$ , versus sulfasalazine group. \$ $p < 0.05$ , versus MSCs group. mRNA: messenger RNA; S1P: sphingosine-1-phosphate; SphK1: sphingosine kinase; SPL: sphingosine-1-phosphate lyase [Color figure can be viewed at [wileyonlinelibrary.com](http://wileyonlinelibrary.com)]



Shi, Wang, Zhu, & Zhao, 2016). In this experiment, coadministration of ASCs and sulfasalazine significantly reversed the downregulation of antiapoptotic protein, Bcl-2, and upregulation of apoptotic protein, Bax after induction of colitis.

Our results suggest that the therapeutic and regenerative effects of coadministration of ASCs and sulfasalazine may be partially explained by downregulation of proinflammatory cytokines levels, inhibition of the NF- $\kappa$ B pathway activation, and moderation of apoptosis occurrence.

The obtained data suggest that combination therapy with MSCs and sulfasalazine (as a reference drug in IBD treatment) may offer potent synergistic therapeutic, anti-inflammatory and colon reconstructive effects and can be used to improve colitis symptoms in IBD patients.

## 5 | CONCLUSION

In conclusion, we have demonstrated that combination treatment with ASCs along with low dose of sulfasalazine exhibited significant anti-inflammatory and regenerative effects than either monotherapy in the mouse model of colon inflammation. The current study suggests that concurrent administration of ASCs and sulfasalazine may be useful to improve clinical outcome of IBD treatment. These results may be used to develop new cell-based therapeutic approaches for colitis.

It is important to note that the implications of the present findings are not exclusively limited to colitis, as the observed synergy effect could also potentiate the individual effect of each component in other inflammatory conditions that are targeted by this treatment (such as rheumatoid arthritis); therefore, it subsequently plays a crucial role in the field of inflammatory diseases.

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## CONFLICTS OF INTEREST

The authors declare that there are no conflicts of interest.

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